SPECIFICATION

ABC TRANSPORTER AND GENE CODING FOR THE SAME

Technical Field

The present invention relates to a novel ABC transporter and a gene coding for a protein that is a constituent of the ABC transporter. The gene can be utilized for breeding of a microorganism showing modified transport of amino acids across a cell membrane and so forth.

Background Art

There are several mechanisms are known for transport of substances such as an amino acids or ions through cell membranes. As one of such mechanisms, the ATP-binding cassette superfamily (ABC transporters) is known (C.F. Higgins, *Ann. Rev. Cell Biol.*, *8*, 67 (1992)).

The ATP-binding cassettes constitute a group of proteins having an ATP-binding domain including a transmembrane domain. Their physiological function is primarily uptake of substances into a cell, but the ATP-binding cassette is considered to also participate in excretion of substances to some extent. In bacteria, they usually contain, as constituents, membrane proteins

(membrane components), proteins that are present inside the membrane and have the ATPase activity, and binding proteins that are present outside the membrane and bound to substances. The membrane proteins and proteins having the ATPase activity form a polymer complex. It is said that the substance excretion system lacks a binding protein bound to a substance to be transported (Reizer, J. *et al.*, *Prot. Sci. 1*, 1326 (1992)).

Since the ABC transporters or constituents thereof are involved in transport of substances, it is considered that characteristics of a cell concerning substance transport can be modified by modifying expression of genes coding for them.

Structures of various ABC transporter genes in bacteria such as *Escherichia coli* have been analyzed, and it is known that each gene coding for constituent of an ABC transporter forms an operon. In coryneform bacteria, however, most of genes coding for ABC transporters or constituents thereof involved in transport of amino acids across membranes remain unknown.

Disclosure of the Invention

The inventors of the present invention cloned a gene coding for an enzyme involved in one of L-glutamic acid biosynthetic pathways, glutamine-oxoglutarate aminotransferase (also referred to as glutamate synthase,

abbreviated as "GOGAT" hereinafter) for the purpose of breeding of coryneform bacteria producing L-glutamic acid. In this process, the inventors accidentally found that a DNA fragment containing a gene coding for GOGAT (*gltBD*) contained a gene coding for an ABC transporter considered to be involved in transport of amino acids, and thus accomplished the present invention.

That is, the present invention provides a protein, which is a constituent of ABC transporter, and a DNA coding for it.

A first constituent of ABC transporter according to the present invention is a protein defined in the following (A) or (B):

(A) a protein which has the amino acid sequence of SEQ ID NO: 8 shown in Sequence Listing;

(B) a protein which has the amino acid sequence of SEQ ID NO: 8 shown in Sequence Listing including substitution, deletion, insertion, addition or inversion of one or several amino acids, and constitutes an ABC transporter.

A second constituent of ABC transporter according to the present invention is a protein defined in the following (C) or (D):

(C) a protein which has the amino acid sequence of SEQ ID NO: 9 shown in Sequence Listing;

(D) a protein which has the amino acid sequence of SEQ ID NO: 9 shown in Sequence Listing including

substitution, deletion, insertion, addition or inversion of one or several amino acids, and has ATPase activity of ABC transporter.

A third constituent of ABC transporter according to the present invention is a protein defined in the following (E) or (F):

(E) a protein which has the amino acid sequence of SEQ ID NO: 10 shown in Sequence Listing;

(F) a protein which has the amino acid sequence of SEQ ID NO: 10 shown in Sequence Listing including substitution, deletion, insertion, addition or inversion of one or several amino acids, and constitutes an ABC transporter.

The present invention also provides DNAs coding for the aforementioned proteins that are constituents of ABC transporter.

The present invention further provides an operon coding for an ABC transporter.

Hereafter, the present invention will be explained in detail.

The DNA of the present invention was found from *Brevibacterium lactofermentum* as an ORF present in the neighborhood of the *gltBD* gene and can be obtained as follows.

PCR (polymerase chain reaction) is performed by using chromosomal DNA of *Brevibacterium lactofermentum* such as *Brevibacterium lactofermentum* ATCC13869 as a

template and primers having nucleotide sequences of regions in the *gltBD* genes of *Escherichia Coli* K-12 (*Gene, vol. 60*, pp.1-11 (1987) and yeast (*Saccharomyces cerevisiae*, GenBank Accession No. X89221) exhibiting high homology, for example, those having nucleotide sequences of SEQ ID NOS: 1 and 2 shown in Sequence Listing, to obtain a DNA fragment of about 1.4 kb. *Brevibacterium lactofermentum* ATCC13869 can be obtained from ATCC (the American Type Culture Collection: 12301 Parklawn Drive, Rockville, Maryland 20852, United States of America).

Subsequently, colony hybridization of a chromosomal DNA library of *Brevibacterium lactofermentum* ATCC13869 is performed by using the PCR-amplified fragment obtained as described above as a probe to obtain a DNA fragment hybridizable with the probe. Thus, the DNA of the present invention can be obtained together with the *gltBD* gene. If chromosomal DNA digested with *Hin*dIII is used in the preparation of the chromosomal DNA library, the DNA fragment can be obtained as a fragment of about 14 kb in length.

The above DNA fragment contains the *gltBD* gene and two open reading frames (ORFs) downstream the *gltBD* gene in the inverted direction with respect to the *gltBD* gene from the end. These ORFs correspond to the second ORF and third ORF, respectively, among the ORFs included in the nucleotide sequence of SEQ ID NO: 7.

As shown in examples described later, it is possible that the aforementioned two ORFs form an operon together with another ORF that exists upstream from them. This ORF corresponds to the first ORF among the ORFs included in the nucleotide sequence of SEQ ID NO: 7. This first ORF can be obtained as a DNA fragment of about 1.8 kb by PCR using chromosomal DNA of *Brevibacterium lactofermentum*, for example, the *Brevibacterium lactofermentum* ATCC13869, as a template and nucleotide sequences of SEQ ID NOS: 5 and 6 shown in Sequence Listing as primers. In this DNA fragment, a region estimated to be a promoter region exists in the upstream of the target ORF.

The nucleotide sequence shown in SEQ ID NO: 7 is obtained by ligating a nucleotide sequence (1.3 kb) in the aforementioned DNA fragment of about 14 kb with a nucleotide sequence (1.1 kb) in the aforementioned DNA fragment of about 1.8 kb.

Since the nucleotide sequences of the above ORFs and nucleotide sequences of flanking regions have been revealed, the above ORFs can also be obtained by PCR using oligonucleotides prepared based on such nucleotide sequences as primers.

Usual methods well known to those skilled in the art can be employed for preparation of chromosomal DNA, construction of chromosomal DNA library, hybridization, PCR, preparation of plasmid DNA, digestion and ligation

of DNA, transformation, design of oligonucleotides to be used as primers and so forth. These methods are described in Sambrook, J., Fritsch, E.F., Maniatis, T., Molecular Cloning, Cold Spring Harbor Laboratory Press, 1.21 (1989) and so forth.

The aforementioned second ORF and amino acid sequence encoded thereby were compared with known sequences for homology. The used databases were EMBL and SWISS-PROT. As a result, these sequences exhibited homology to already reported ATPase proteins constituting ABC transporters responsible for transport of the amino acids listed in Table 1 and genes coding for them. It is possible that the three ORFs containing these sequences form an operon.

Table 1

Gene	Substance to be tranported	Origin	Reference	Homology
artP	Arginine	*E. coli*	J.Bacteriol.175: 3687-3688 (1993)	31.0%
artP	Arginine	*Haemophilus Influenzae*	Science 269: 496-512 (1995)	31.8%
glnQ	Glutamine	*Bacillus Stearothermophilus*	J.Bacteriol.173: 4877-4888 (1991)	35.4%
glnQ	Glutamine	*E. coli*	Mol.Gen.Genet.205: 260-269 (1986)	33.5%
GltL	Glutamic acid/ Aspartic acid	*E. coli*	GeneBank Accession No.U10981	33.5%
gltL	Glutamic acid/ Aspartic acid	*Haemophilus influenzae*	Science 269: 496-512 (1995)	31.2%
gluA	Glutamic acid	*Corynebacterium glutamicum*	J.Bacteriol.177: 1152-1158	34.4%
hisP	Histidine	*E. coli*	Nature 298: 723-727 (1982)	33.0%
hisP	Histidine	*Salmonella typhimurium*	Nucleic acids Res.15: 8568-8568	34.4%

The gene coding for a constituent of ABC transporter according to the present invention may be one coding for an ATP-binding protein including substitution, deletion, insertion, addition or inversion of one or several amino acids at one or a plurality of positions so long as characteristics of the encoded protein are not deteriorated. The number meant by the term "several" used herein may vary depending on locations of amino acid residues in the three-dimensional structure of proteins and kinds of amino acid residues. This is due to the fact that there are highly analogous amino acids among amino acids such as isoleucine and valine, and difference among such amino

acids does not substantially affect the three-dimensional structure of proteins.

Such a DNA encoding a protein substantially the same as a constituent of ABC transporter as mentioned above can be obtained by modifying a nucleotide sequence by, for example, site-directed mutagenesis so that the amino acid residues of a specific site should include substitution, deletion, insertion, addition or inversion. Such a modified DNA as mentioned above can also be obtained by an already known mutagenesis treatment. Examples of the mutagenesis treatment include *in vitro* treatment of DNA coding for each protein with hydroxylamine etc., treatment of a microorganism having DNA coding for each protein, for example, genus *Escherichia*, by ultraviolet irradiation or with a mutagenizing agent used for usual mutagenesis treatment such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG) or nitrous acid.

The substitution, deletion, insertion, addition or inversion of nucleotides described above also includes mutations (mutant or variant) that naturally occurring due to individual difference, difference in species or genera of a microorganism having each constituent.

A DNA coding for substantially the same protein as a constituent of ABC transporter can be obtained by expressing DNA having such a mutation as described above in an appropriate cell, and examining characteristics of

an expressed product. A DNA coding for substantially the same protein as a constituent of ABC transporter can also be obtained by isolating a DNA hybridizable with a nucleotide sequence coding for each constituent or a probe prepared from such a nucleotide sequence, for example, the nucleotide sequence of nucleotide numbers 1117 to 1725 in SEQ ID NO: 7 or a probe prepared from this nucleotide sequence, for ATPase under a stringent condition, and coding for a protein having the characteristics of the constituent from a DNA coding for each protein having mutation or from a cell harboring it. The "stringent condition" referred to herein is a condition under which a so-called specific hybrid is formed, but a non-specific hybrid is not formed. It is difficult to clearly define this condition by using numerical values. However, for example, the stringent condition includes a condition under which two of DNAs having high homology, for example, two of DNAs having homology of not less than 40% are hybridized with each other, but two of DNAs having homology lower than the above level are not hybridized with each other. Alternatively, the stringent condition is exemplified by a hybridization condition represented by salt concentrations of 1 x SSC, 0.1% SDS, preferably 0.1 x SSC, 0.1% SDS, at 60°C, which is an ordinary condition of washing in Southern hybridization.

Those genes hybridizable under the condition as

described above include those having a stop codon generated in the genes, and those having no activity due to mutation of the active center. However, such mutant genes can be easily removed by using a commercially available activity expression vector to examine the characteristics of the expressed product.

The DNA coding for a constituent of ABC transporter according to the present invention and an operon of ABC transporter (hereafter, these may be referred to simply as "gene of the present invention") can be utilized in breeding of coryneform bacteria. That is, since the ABC transporter of the present invention or a constituent thereof is considered to be involved in transport of amino acids, characteristics of a cell concerning transport of amino acids can be modified by modifying expression of these genes.

Coryneform bacteria to which the present invention is applicable include those bacteria having been hitherto classified into the genus *Brevibacterium* but united into the genus *Corynebacterium* at present (*Int. J. Syst. Bacteriol.*, *41*, 255 (1981)), and include bacteria belonging to the genus *Brevibacterium* closely relative to the genus *Corynebacterium*. Examples of such coryneform bacteria are mentioned below.

Corynebacterium acetoacidophilum

Corynebacterium acetoglutamicum

Corynebacterium alkanolyticum

Corynebacterium callunae

Corynebacterium glutamicum

Corynebacterium lilium (*Corynebacterium glutamicum*)

Corynebacterium melassecola

Corynebacterium thermoaminogenes

Corynebacterium herculis

Brevibacterium divaricatum (*Corynebacterium glutamicum*)

Brevibacterium flavum (*Corynebacterium glutamicum*)

Brevibacterium immariophilum

Brevibacterium lactofermentum (*Corynebacterium glutamicum*)

Brevibacterium roseum

Brevibacterium saccharolyticum

Brevibacterium thiogenitalis

Brevibacterium album

Brevibacterium cerium

Microbacterium ammoniaphilum

Specifically, the following strains can be exemplified.

Corynebacterium acetoacidophilum ATCC 13870

Corynebacterium acetoglutamicum ATCC 15806

Corynebacterium alkanolyticum ATCC21511

Corynebacterium callunae ATCC 15991

Corynebacterium glutamicum ATCC 13020, 13032, 13060

Corynebacterium lilium (*Corynebacterium glutamicum*) ATCC 15990

Corynebacterium melassecola ATCC 17965

Corynebacterium thermoaminogenes AJ12340 (FERM BP-1539)

Corynebacterium herculis ATCC13868

Brevibacterium divaricatum (*Corynebacterium glutamicum*) ATCC 14020

Brevibacterium flavum (*Corynebacterium glutamicum*) ATCC 13826, ATCC 14067

Brevibacterium immariophilum ATCC 14068

Brevibacterium lactofermentum (*Corynebacterium glutamicum*) ATCC 13665, ATCC 13869

Brevibacterium roseum ATCC 13825

Brevibacterium saccharolyticum ATCC 14066

Brevibacterium thiogenitalis ATCC 19240

Brevibacterium album ATCC15111

Brevibacterium cerium ATCC15112

Microbacterium ammoniaphilum ATCC15354

Methods of modifying a gene coding for an ABC transporter or a constituent thereof include amplification or disruption of the gene. The gene or the like can be amplified by transforming a coryneform bacterium with a recombinant vector obtained by ligating the gene to a vector such as a plasmid. At this time, amplification efficiency can be improved by using a multiple copy type vector. Examples of such a vector

include plasmids autonomously replicable in coryneform bacterium including those mentioned below.

pAM330 (refer to Japanese Patent Laid-Open (Kokai) No. 58-67699)

pHM1519 (refer to Japanese Patent Laid-Open No. 58-77895)

pAJ655 (refer to Japanese Patent Laid-Open No. 58-192900)

pAJ611 (refer to Japanese Patent Laid-Open No. 58-192900)

pAJ1844 (refer to Japanese Patent Laid-Open No. 58-192900)

pCG1 (refer to Japanese Patent Laid-Open No. 57-134500)

pCG2 (refer to Japanese Patent Laid-Open No. 58-35197)

pCG4 (refer to Japanese Patent Laid-Open No. 57-183799)

pCG11 (refer to Japanese Patent Laid-Open No. 57-183799)

Coryneform bacteria can be transformed by the electric pulse method (refer to Japanese Patent Laid-Open No. 2-207791).

The gene can also be amplified by allowing multiple copies of the gene of the present invention to exist on chromosomal DNA of a host such as those mentioned above. Multiple copies of a target gene can

be introduced into chromosomal DNA of coryneform bacterium by homologous recombination utilizing multiple copies of sequences existing on chromosomal DNA as targets (Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press (1972); Matsuyama, S. and Mizushima, S., *J. Bacteriol.*, *162*, 1196 (1985)). As sequences of which multiple copies exist on the chromosomal DNA, repetitive DNA and inverted repeats that exist at an end of transposable element can be used. As disclosed in Japanese Patent Laid-open No. 2-109985, it is also possible to insert the target gene into transposon, and allow it to transfer to introduce multiple copies thereof into the chromosomal DNA.

Further, expression of the gene can be modified by replacing an expression regulatory sequence of the gene originally present on a chromosome, such as a promoter, with a stronger one or one having weak functions.

Moreover, gene disruption methods by homologous recombination have already been established, and the gene can be disrupted by a method using linear DNA or a temperature sensitive plasmid.

Best Mode for Carrying out the Invention

Hereafter, the present invention will be explained in more detail with reference to the following examples.

(1) Cloning of *gltBD* gene of *Brevibacterium lactofermentum* ATCC13869

A region of *gltB* gene products of *Escherichia coli* and yeast showing high homology for amino acid sequence was selected, and a nucleotide sequence was deduced from the sequence, oligonucleotides shown as SEQ ID NOS: 1 and 2 were synthesized. Separately, chromosomal DNA of *Brevibacterium lactofermentum* ATCC13869 was prepared by using a Bacterial Genomic DNA Purification Kit (produced by Advanced Genetic Technologies Corp.). PCR was performed by using this chromosomal DNA as a template and the oligonucleotides as primers under the standard reaction conditions described in "PCR Technology", p. 8, Ed. by Henry Ehrlich, Stockton Press, 1989. The PCR product was subjected to agarose gel electrophoresis, and it was found that a DNA fragment of about 1.4 kb was amplified.

The obtained DNA was sequenced for the nucleotide sequences of the both ends by using the oligonucleotides of SEQ ID NOS: 1 and 2. The nucleotide sequencing was performed according to the method of Sanger (*J. Mol. Biol., 143,* 161 (1980)) by using a DNA Sequencing Kit (produced by Applied Biosystems Co.). The determined nucleotide sequence was translated into an amino acid sequence, and compared with an amino acid sequence deduced from the *gltB* gene of *Escherichia coli* and yeast. As a result, high homology was observed. Therefore, it

was determined that the DNA fragment amplified by the PCR should be a part of the gltB gene of *Brevibacterium lactofermentum* ATCC13869. By using this PCR-amplified DNA fragment as a probe and a DIG DNA Labeling and Detection Kit (produced by Boehringer Manheim), fragments obtained by digesting chromosomal DNA of *Brevibacterium lactofermentum* ATCC13869 prepared by the above method with *Eco*RI, *Bam*HI, *Hin*dIII, *Pst*I or *Sal*I (produced by Takara Shuzo Co., Ltd.) were subjected to Southern hybridization in a conventional manner. AS a result, it was found that a fragment of 14 kb digested with *Hin*dIII was hybridized with the probe DNA.

Then, the *Hin*dIII fragment of chromosomal DNA of *Brevibacterium lactofermentum* ATCC13869 prepared in a conventional manner was subjected to agarose electrophoresis and a DNA fragment of about 10 kb or longer was recovered by using glass powder. The recovered DNA fragments and vector pMW219 (produced by Nippon Gene) digested with a restriction enzyme, *Hin*dIII (produced by Takara Shuzo Co., Ltd.), were ligated by using a ligation kit (produced by Takara Shuzo Co., Ltd.), and used for transformation of competent cells of *Escherichia coli* JM109 (produced by Takara Shuzo Co., Ltd.). The transformant strains were plated on L medium (10 g/L of Bacto trypton, 5 g/L of Bacto yeast extract, 5 g/L of NaCl, and 15 g/L of agar, pH 7.2) containing 10 μg/ml of IPTG (isopropyl-â-D-thiogalactopyranoside), 40

μg/ml of X-Gal (5-bromo-4-chloro-3-indolyl-â-D-galactoside) and 25 μg/ml of kanamycin, and cultured overnight. Then, the appeared white colonies were picked up and separated into single colonies to obtain about 1,000 transformants.

Plasmids were prepared from the obtained transformant strains by using the alkaline method (Text for Bioengineering Experiments, Edited by the Society for Bioscience and Bioengineering, Japan, p.105, Baifukan, 1992). PCR was performed under the above conditions by using as primers synthetic oligonucleotides of nucleotide sequences shown as SEQ ID NOS: 3 and 4, which were prepared based on the sequenced portion in the DNA used as a probe, and the plasmids as a template. Then, there was selected a transformant harboring a plasmid with which an amplified fragment having the same length as the DNA fragment amplified by PCR using these primers and chromosome of *Brevibacterium lactofermentum* ATCC13869 as a template, that is, about 1.3 kb, could be obtained.

(2) Sequencing of DNA fragment containing *Brevibacterium lactofermentum* ATCC13869 *gltBD* gene for total nucleotide sequence and isolation of ABC transporter gene

The plasmid DNA prepared by the alkaline method from the transformant obtained in the above (1) contained a DNA fragment of about 14 kb derived from a

Brevibacterium lactofermentum ATCC13869 chromosome. The DNA fragment of about 14 kb derived from the *Brevibacterium lactofermentum* ATCC13869 chromosome in the obtained plasmid was sequenced for the total nucleotide sequence in the same manner as the method described above. As a result, it was found that, while the obtained DNA fragment contained the *gltBD* gene in the full length, it also contained two open reading frames of 500 bps or longer downstream from the *gltBD* gene in an inverted direction from the end and a sequence estimated to be a terminater downstream from these open reading frames. However, since these open reading frames lacked a promoter region, a region upstream from them was cloned as described below.

The region was cloned from a DNA fragment obtained through digestion of chromosome of *Brevibacterium lactofermentum* ATCC13869 with a restriction enzyme *Bam*HI by using primers of SEQ ID NOS: 5 and 6 shown in Sequence Listing and an LA PCR in vitro cloning Kit (produced by Takara Shuzo Co., Ltd.). As a result of PCR performed by using the aforementioned primers, a DNA fragment of about 1.8 kb was amplified, and hence this DNA fragment was sequenced for the nucleotide sequence in the same manner as described above. As a result, it was found that the amplified DNA fragment contained an open reading frame for about 350 amino acids located upstream from the aforementioned two open reading frames

and a region estimated to be a promoter region further upstream from it. Therefore, it is possible that these three open reading frames consititute an operon.

Nucleotide sequences of these open reading frames are shown in SEQ ID NO: 7 in Sequence Listing. Amino acid sequences of products deduced from the nucleotide sequences were also shown in SEQ ID NO: 7 in Sequence Listing. Among these, the nucleotide numbers 1 to 1101 represent the first open reading frame, the nucleotide numbers 1117 to 1725 represent the second open reading frame and the nucleotide numbers 1759 to 2367 represent the third open reading frame. A methionine residue present at the N-terminus of the protein encoded by each open reading frame was derived from the initiation codon. It is well known that such a methionine residue may be usually irrelevant to function of the protein and eliminated by the action of peptidase after the translation. In the case of the aforementioned proteins, the methionine residue at the N-terminus may also be eliminated. Further, since the promoter region and terminater sequence estimated above were obtained just as a result of computarized analyses, it is possible that open reading frames may be present upstream or downstream from them and expressed together with them in fact.

The nucleotide sequences and amino acid sequences were compared with known sequences for homology. The

used database were EMBL and SWISS-PROT. As a result, it was found that DNA shown as SEQ ID NO: 7 in Sequence Listing and proteins encoded by it were novel genes and proteins for bacteria belonging to the genus *Corynebacterium*. It was found that, among these, the second open reading frame and the protein encoded by it showed high homology to the already reported ATP-binding proteins of ABC transporters and the genes coding for them, and it was a gene coding for an ATP-binding protein that was novel for bacteria belonging to the genus *Corynebacterium*.

Industrial Applicability

According to the present invention, constituents of ABC transporters of *Brevibacterium lactofermentum* and DNA coding for them are provided. The genes of the present invention can be utilized for breeding of coryneform bacteria.